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Burn Wound Bacteria Susceptibility to a Novel Antimicrobial Compound

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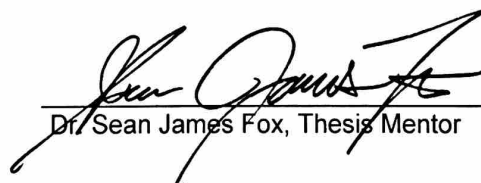
Burn Wound Bacteria Susceptibility to a Novel Antimicrobial Compound

By

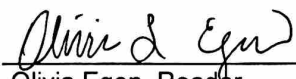
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An Undergraduate Thesis Submitted in Partial Fulfillment
of the Requirements for the
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Abstract

A breach of the skin barrier, due to a burn wound, facilitates colonization by various microorganisms. Burn wounds can become colonized from the patients' own skin flora, respiratory tract, or with exogenous bacteria from the environment. Strategies to treat burn wound infections are multipronged: removal of the infected necrotic tissue, wound dressing to protect the damaged area, and treatment with specific antimicrobials to prevent reinfection. The development of chronic infections, which could potentially lead to sepsis, depends largely on how well the microorganisms form biofilms within the wound. There are numerous antimicrobial gels and antibiotics that help prevent a burn wound from becoming infected, as well as, eliminate an already infected burn wound. However, global antibiotic resistance by microorganisms to these treatments has greatly increased, and it is imperative that new antimicrobial agents be formulated before infections become untreatable. *Staphylococcus*, *Pseudomonas*, *Acinetobacter*, *Escherichia*, *Candida*, *Citrobacter*, and *Klebsiella* are common causative agents of burn wound infections and are becoming increasingly resistant to antimicrobial medications. A newly synthesized antimicrobial gel compound (AGC) has shown promise in preventing growth of various bacteria and fungi commonly associated with burn wound infections. This study evaluated the activity of the AGC on a panel of Gram-positive and Gram-negative bacteria, and the fungi, *Candida albicans*, which represent the top ten causative infectious agents of burn wounds. The AGC reduced, to varying degrees, the microorganism's growth, cell viability, and cellular metabolism. This novel antimicrobial compound shows

promising potential as an effective option for prevention and treatment of infections in burn wound victims to avoid sepsis.

Introduction

Burn Wounds

Burn wounds are extremely different from other wounds, to the point that a separate medical specialty has been designed to manage them. Heat from burns not only damages skin locally, but also causes generalized effects on the body. The degree of the burn has an enormous influence on how life-threatening the situation is (12). Acute burn wounds are a complex and evolving injury, and extensive burns produce systemic consequences. Any adult burn over 15% and pediatric burn over 10% will produce a hypovolemic shock due to the increase in capillary permeability resulting in an extensive loss of plasma and whole blood loss. Burns can involve all layers of skin as well as structures that are beneath such as muscle, bone, blood vessels, nerves, and hair follicles (13). Burn wounds are classified into three different categories: first-degree burn, second-degree burn, and third degree burn. In first-degree burns, or epithelial burns, the skin is erythematic without vesication. Second-degree burns involve the epidermis with variable thickness of the dermis. Second-degree burns can be further divided into second-degree superficial and second-degree deep. Second-degree superficial burns have visible vesication and inflammation, but only on the superficial portion of the dermis. Second-degree deep burns penetrate the deep reticular dermis where eschar formation is seen. Third-degree burns, or full thickness burns, produce eschar (12). Eschar is normally a dry, dark scab or dead skin shedding.

Physicians and other healthcare personnel are more involved with the treatment of patients with burn wounds than any other traumatic wound.

Microorganisms Associated with Burn Wounds

Bacterial colonization reaches as high as 86.6% of the isolates within the first week of infection. Gram-negative microorganisms are more predominant; however, *Staphylococcus aureus* is the most prevalent organism in the first week following the burn and is about 30% of the bacterial isolates (7). By the third week, *Staphylococcus aureus* is closer to 10% of isolates while *Pseudomonas aeruginosa* reaches about 30% of the isolates. This demonstrates how crucial it is to treat a burn wound according to the specific microorganism(s) that colonize it because microorganisms have different growth patterns. Thus, contamination of the burn wound is the guideline rather than an exception in burn wounds (7). Biofilms can become established within wounds in 5 hours and mature by 10 hours; therefore, the faster the wound acquires medication the better the outcome for the individual (6, 8). Burn patients are at an extremely high risk for infection, and about 75% of all burn wound deaths are related to infection (10). *Staphylococcus*, *Streptococcus*, *Pseudomonas*, *Acinetobacter*, *Escherichia*, *Candida*, *Citrobacter*, and *Klebsiella* are common causative agents of burn wound infections with increasing resistance to many antimicrobial medications. For instance, *Streptococcus pyogenes* was the most frequent cause of burn wound infections, which induced sepsis, early in the last century. As of now, *Staphylococcus aureus* and *Pseudomonas aeruginosa* have become the most frequent isolated organisms from wounds caused by burns due to their ability to effectively evade certain medical treatments (7).

Current Treatments for Infected Burn Wounds

Since ancient time, local and systemic remedies have been used for burn wounds. Death associated with extensive burns is mainly due to wound infection as the patient becomes immunocompromised from the burn (12). The gels that exist today do fight infection to decrease the number of deaths caused by burns, but with increasing difficulty due to resistance. One gel, *Aloe vera inner gel*, has made an astonishing impact as it can be used as an antimicrobial, anti-inflammatory, lipid and glucose lowering agent, and has antioxidant functions. This gel showed remarkable inhibition of *H. Pylori* and *E. Coli* strains (1). Next Science Wound Gel Technology (NxtSc), is another topical agent that is designed to kill planktonic bacteria, but this gel also can penetrate biofilms and kill the bacteria within. It works against various bacterial infections that can lead to life threatening sepsis. The NxtSc has proven to inhibit the growth of *S. aureus* and *P. aeruginosa* in infected wounds and eliminates other gram-positive and gram-negative organisms from infected wounds (8). Guar gum is a new antimicrobial peptide delivery system that helps fight against diabetic foot ulcers, which was distinguishable in its ability to fight against *Staphylococcus aureus* in these foot ulcers (11). Non-aqueous glycerol monolaurate gel (GML) is another gel that fights *Staphylococcus aureus* infections and is useful as a potent antibacterial and anti-inflammatory compound for infections. GML is time-dependent when removing biofilms, with higher concentrations acting at earlier time periods (14). Chitosan, a cationic natural polymer, can be used to prevent and treat burn wounds because of its ability to deliver extrinsic antimicrobial agents to the site of infection and its natural antimicrobial properties (4). Some synthetic skin substitutes that aid in the antimicrobial healing process include Duoderm, Omniderm, Epigard, Biobrane, and

Dermagraft. Commonly known topical antimicrobial antibiotics include Mafenide acetate, Bacitracin, and Neosporin (3). Other types of interventions include burn wound dressings to treat superficial and partial thickness burns. These dressings range from hydrocolloid and polyurethane dressings to hydrogel and silicon-coated dressings (13). Though these are all excellent, their effects are becoming nonexistent due to certain resistant bacteria. We present here, an antimicrobial gel compound (AGC) that affects microorganisms commonly associated with burn wounds. This new compound has proven to eliminate, or inhibit, specific microbial growth of microorganisms such as *Escherichia coli*, *Candida albicans*, *Citrobacter freundii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Serratia marcescens*, and *Enterobacter cloacae*. AGC not only decreased the microbial cell density and metabolism, but also decreased the CFU's on LB agar plates.

Materials and Methods

Strains & Culture Conditions

Bacteria utilized for experiments are listed in Table 1. All bacterial strains were purchased from American Type Culture Collection (ATCC). Media for propagation and experiments involving these strains was LB Miller broth (LB), a 10% (w/v) AGC broth with LB, or on LB agar plates.

Microorganism	ATCC strain	Type of microorganism	Gram reaction	Oxygen requirement	Biofilm Former
<i>Candida albicans</i>	10213	Eukaryote	N/A	Facultative anaerobe	Yes
<i>Citrobacter freundii</i>	8090	Prokaryote	Negative	Facultative anaerobe	Yes
<i>Enterobacter cloacae</i>	23355	Prokaryote	Negative	Facultative anaerobe	Yes
<i>Escherichia coli</i>	25922	Prokaryote	Negative	Facultative anaerobe	Yes
<i>Klebsiella pneumoniae</i>	13883	Prokaryote	Negative	Facultative anaerobe	Yes
<i>Pseudomonas aeruginosa</i>	10145	Prokaryote	Negative	Facultative aerobe	Yes
<i>Serratia marcescens</i>	13880	Prokaryote	Negative	Facultative anaerobe	Yes

Table 1. Strains used in experiments

Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Strains were inoculated in LB broth for 18 hours, at 37°C, with shaking (255rpm). These seed cultures were used to inoculate, into varying concentrations of AGC-LB broth (0%-10% w/v), at a final concentration of 1×10^6 cells/ml. These tubes were then incubated an additional 24

hours. The MIC was determined visually by the lack of turbidity. Once the MIC was determined aliquots from all AGC-LB broth tubes were plated onto LB agar plates. MBC was determined as the lowest percentage plate that was void of growth.

Growth, Metabolism, and Viability Inhibition Assay

Strains were inoculated into LB broth for 18 hours, at 37°C, with shaking (255rpm). These seed cultures were then used to inoculate LB broth (control) or 10% AGC-LB broth (experimental) at a final concentration of 1×10^6 cells/ml. These tubes were then incubated an additional 24 hours with shaking (255rpm). The cultures were added (100µl) to 96 well plate rows. Rows 1-6 were used to measure the optical density (OD₆₀₀) measurements of growth. Rows 7-12 were used for optical density (OD₅₇₀) measurements of metabolism of MTT. Briefly, 5µg/ml of MTT was added to wells 7-12 and incubated at 37°C for 30 minutes. Acidic Isopropanol was added to stop the reaction and optical density was then determined. Controls of LB broth only and 10% AGC-LB only (no bacteria) were also added to the 96 well plate and treated, as above, to correct for background and to act as a control. For determination of cell viability each condition was serially diluted, plated on LB agar, and colony forming units (CFU) were enumerated.

Results and Discussion

AGC shows varying inhibitory MICs on bacteria commonly associated with burn wounds.

The minimum inhibitory concentration (MIC) is the lowest concentration of an antimicrobial that will inhibit the visible growth of bacteria after incubation. Of the microorganisms tested the concentration of bacterial cells grown with AGC was reduced when compared to the

bacterial growth in the LB broth (0% AGC) control (**Table 2**). *Candida albicans* and *Citrobacter freundii* displayed the lowest MIC at 3% AGC-LB broth concentrations. *Serratia marscescens* demonstrated less turbidity when compared to the 0% control. However, there was growth in all of the concentrations of AGC tested (0-10%); therefore, MIC was not determined. *Enterobacter cloacae* (4%), *Escherichia coli* (6%), *Klebsiella pneumoniae* (6%), and *Pseudomonas aeruginosa* (5%) all had varying MICs (**Table 2**). There appears to be no defining characteristic that correlates with MIC among the tested microorganisms as there are representatives of prokaryotes and eukaryotes, morphology, Gram classification, and oxygen requirements (**Table 1**). Additional work by our research group has shown that *Staphylococcus aureus* is highly susceptible to the inhibitory effects of the AGC (data not shown).

AGC does not appear to have a cidal effect from the MBC concentrations tested.

The minimum bactericidal concentration (MBC) is the lowest concentration of an antibacterial medication required to kill a specific bacterial strain. Only one of the microorganisms tested, *Escherichia coli* at 9% AGC, had a detectable MBC (**Table 3**). All other microorganisms had detectable growth when plated at all concentration dilutions, though growth was reduced. This observation provides two possible explanations: (1) That AGC has bacteriostatic effects on microorganisms and inhibits only growth, because there is no true MBC within the 10 concentrations tested; therefore, AGC may only serve as a compound that inhibits growth; (2) that an increased percentage of AGC-LB is needed to determine the MBC. This potential explanation may be considered in future experiments, however, we have only been successful in a maximum 15% (w/v) AGC to LB broth formulation. Above this percentage, the AGC does

not suspend in the LB broth. Additionally, rule-of-thumb for experiments is that the vehicle (AGC) does not contribute more than 10% of the total volume. This takes into account effects that any gel compound would impose on bacterial growth due to limiting the bacteria's normal requirements of growth (i.e. oxygen requirements).

AGC inhibits microorganism growth, cell metabolism, and cell viability.

Using the information from the MIC and MBC experiments, it was determined that the optimal concentration for further experiments examining the microorganism's growth, cellular metabolism, and cell viability would be 10% AGC. A high throughput 96-well plate assay was developed to allow for testing of all three conditions simultaneously. Microorganisms were inoculated (1×10^6 cells/ml) and incubated overnight in LB broth with or without 10% AGC. These cultures were then aliquoted equally into rows of 96-well plates. Wells 1-6 were used to obtain optical density (OD₆₀₀) readings for growth analysis. Wells 7-11 were used to obtain optical density (OD₅₇₀) readings for the reduction of MTT to formazan, to measure cell metabolism. Well 12 was untreated and used to serially dilute the sample and plate on LB agar to enumerate CFU data, to measure cell viability. In regards to optical density, or microbial growth density, all microorganisms tested in the presence of 10% AGC displayed significant reduction in optical density (Figure 1). *Escherichia coli* and *Citrobacter freundii* had the largest decreases in optical density, 92% and 75% reduction respectively, as compared to their controls, whereas, *Candida albicans* had the smallest decrease in optical density growth, 29%. Optical density readings only identify the density of growth based on turbidity. Optical density cannot identify the state, alive or dead, of the cells contained within the solution. We therefore turned our attention to

CFU methods of determining the state of the microorganisms. Samples of each of the microbial growth, with and without 10% AGC, were used to dilute out and plate on LB agar plates. In theory, a single viable, alive, microorganism has the ability to grow into a colony. Once colonies are enumerated they can be compared between control and experimental conditions. The bacterial growth on LB plates differed tremendously between the AGC treated microbes and the LB only microbes (see Table 4 for representative photographs). Bacterial growth on the plates containing the LB broth with bacteria had growth up to dilutions of 10^9 , but the plates containing AGC treated bacteria were inhibited and most of the growth, if any, was not found above dilution of 10^3 (**Table 4**). All microorganisms tested displayed decreased CFUs in comparison to their untreated controls. In contrast to the optical density growth results, *Klebsiella pneumoniae* had the largest decrease in CFUs at 90%. *Candida albicans* and *Escherichia coli*, which had the largest optical density growth decrease had a more moderate CFU decrease of 59% and 57%, respectively. This indicates that while *Candida* and *Escherichia* are initially able to grow, shown by the increase in optical density, the AGC inhibits or damages the cells, thus a decrease in viable cells to produce colony forming units is seen. *Pseudomonas aeruginosa*, 41%, and *Serratia marcescens*, 35%, had the smallest decreases in CFU. This correlates with the original MIC and MBC data, in that, the AGC for these two microorganisms, in general, only inhibits their growth. The last assay performed, MTT reduction, tells us more about the metabolism and viability of the microorganisms when treated with AGC. The yellow compound MTT is reduced by actively metabolizing bacteria, via their mitochondria, to produce a purple colored, reduced compound. The optical spectra can then measure the amount of reduced product and when compared to viable untreated controls, can give information as to

the metabolic activity of microorganisms. The cellular metabolism results were dramatic, with all microorganisms having significant reductions in the ability to reduce the measured compound. The metabolism of the bacteria not containing the AGC exhibited little to no inhibition, whereas the bacterial sample containing the gel caused the cells metabolism to come to a near halt with very little activity (**Figure 3**). However, as significant as these results were, we had some interesting observations with this assay. When the MTT was added to the AGC only control (no microorganisms present) we observed an almost immediate purple color change. As this control is absent of bacteria, there should not have been a color change. The MTT assay is based on a reducing reaction and there are a number of compounds and elements that make up the AGC that have reducing activities. Therefore, we cannot accurately draw any conclusions from this experiment on the viability of the microorganisms in regards to their metabolic activity. Further experiments will need to be conducted with different ingredients of the AGC, which cause reducing activity, removed so that better conclusions can be made as to viability in regards to metabolism.

Conclusion

About 2 million fires are reported every year in the United States, resulting in, 1.2 million people burned. In patients with severe burns, which cover more than 40% of the total body surface area (TBSA), 75% of deaths are currently related to sepsis from burn wound infection, other infection complications, and/or inhalation injury (2). There are many bacteria that colonize burn wounds that can potentially lead to septic shock and death such as, *Staphylococcus*, *Pseudomonas*, *Acinetobacter*, *Escherichia*, *Candida*, *Citrobacter*, and *Klebsiella*;

which are becoming more resistant to antimicrobial medications. Due to this increased resistance in microorganisms to current medications and treatments, a new treatment method is essential. The AGC examined in this study shows ability to reduce growth and viability of a variety of microorganisms commonly associated with burn wound infections. The activity of the AGC, at this point, can best be categorized as a bacteriostatic, growth inhibiting, agent. This inhibition is apparent in the low MIC ranges, but continued growth during the MBC ranges that were tested was seen. Supporting this, CFUs, albeit low for some bacteria were still present when using the highest concentration of AGC, 10%, tested. The results of these tests indicate that AGC could potentially be a powerful tool for the prevention of infection of burn wounds or be used to inhibit infected burn wound growth by microorganisms. If AGC is truly a bacteriostatic compound it may not elicit the selective pressure on microorganisms to develop drug resistance. In this case, AGC could prove to be a potentially lifesaving treatment for those individuals suffering from burn wounds.


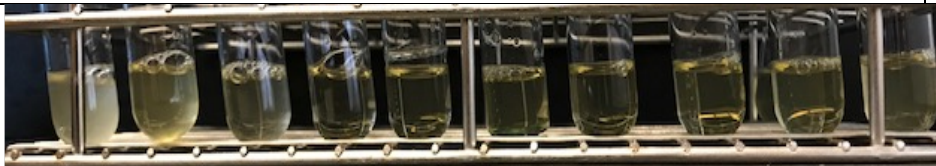
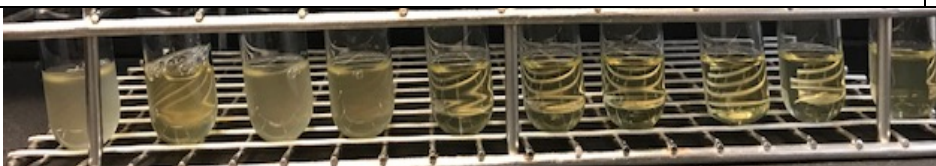
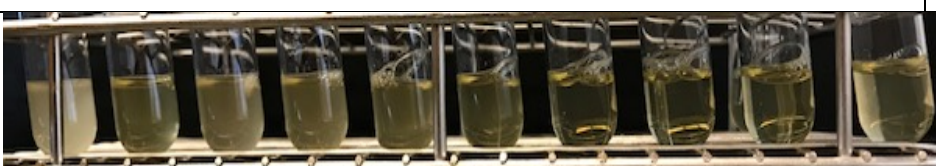
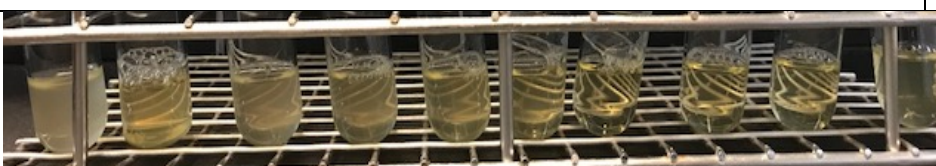
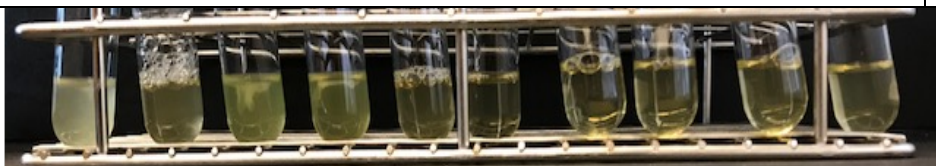

Microorganism	0%	1%	2%	3%	4%	5%	6%	7%	8%	9%	MIC
<i>Candida albicans</i>											3%
<i>Citrobacter freundii</i>											3%
<i>Enterobacter cloacae</i>											4%
<i>Escherichia coli</i>											6%
<i>Klebsiella pneumoniae</i>											6%
<i>Pseudomonas aeruginosa</i>											5%
<i>Serratia marscescens</i>											*

Table 2. Determination of the MIC of bacteria associated with burn wounds when exposed to varying concentrations of AGC.

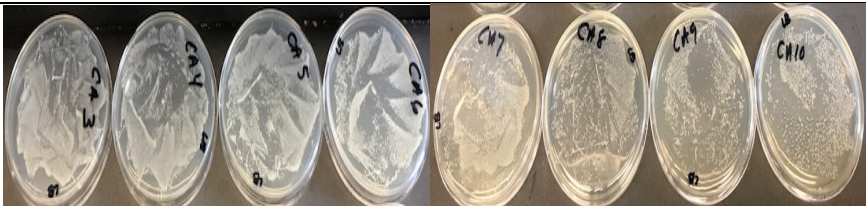

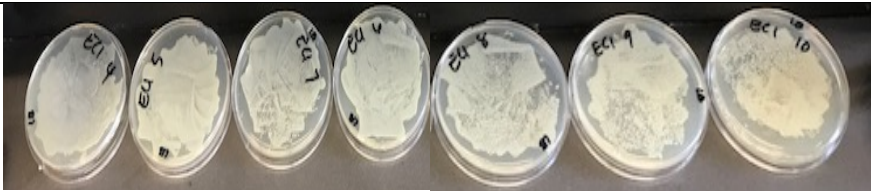
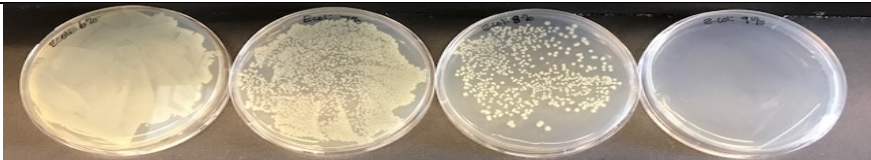
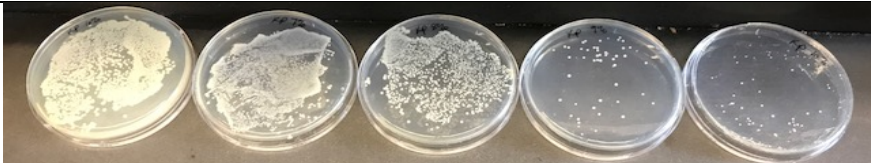
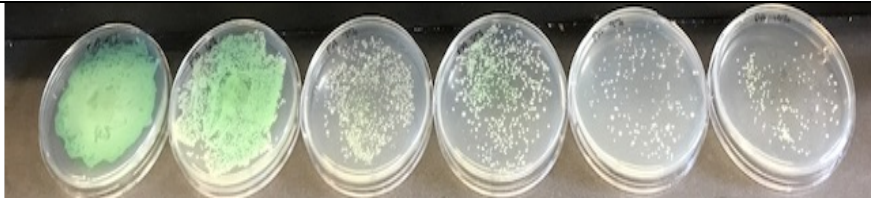
Microorganism		MBC
<i>Candida albicans</i>		>10%
<i>Citrobacter freundii</i>		>10%
<i>Enterobacter cloacae</i>		>10%
<i>Escherichia coli</i>		9%
<i>Klebsiella pneumoniae</i>		>10%
<i>Pseudomonas aeruginosa</i>		>10%
<i>Serratia marcescens</i>	*	>10%

Table 3. Determination of MBC of bacteria associated with burn wounds when exposed to varying concentration of AGC.

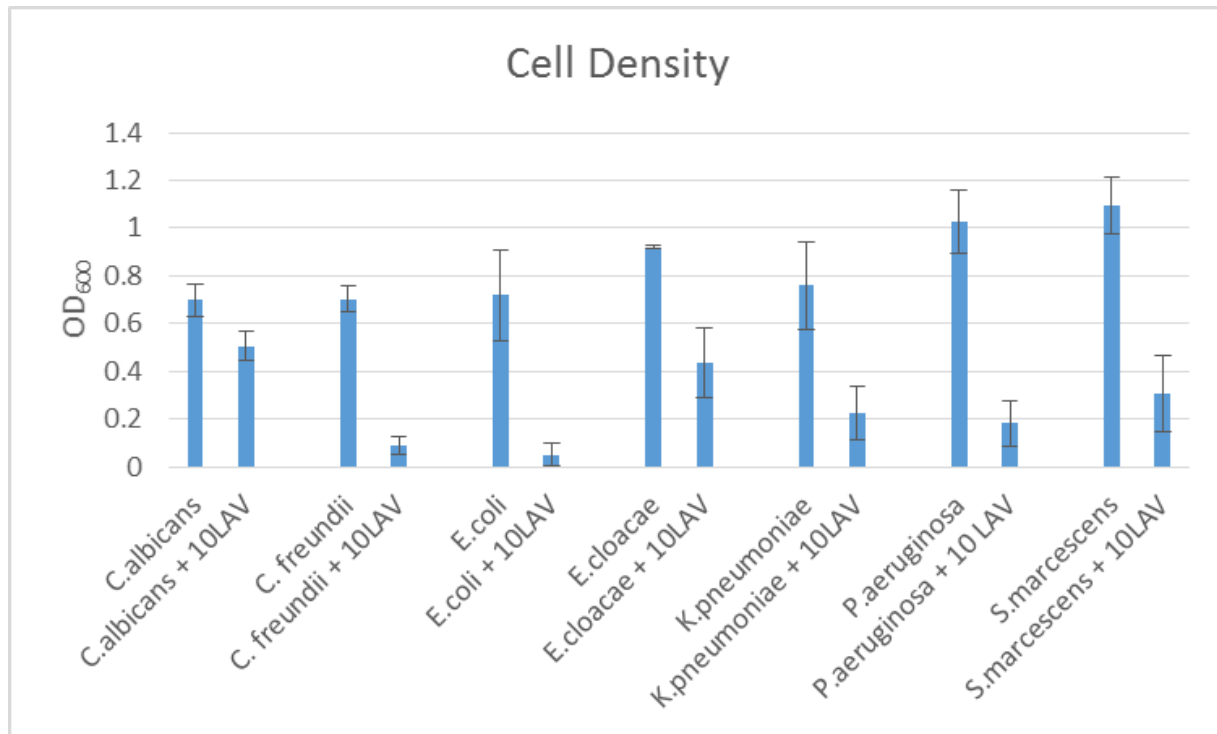


Figure 1. Optical density (OD₆₀₀) graph of microorganism growth with or without 10% AGC.

Standard deviation bars shown.

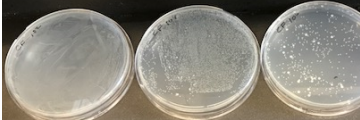
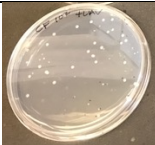
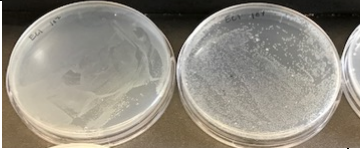
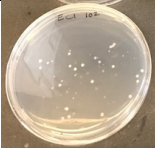


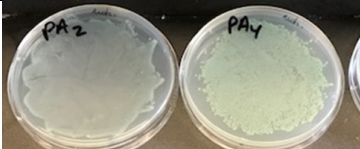

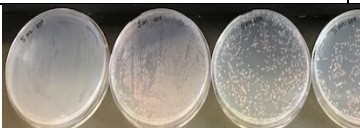

Microorganism	Control (No AGC)	Experimental (10% AGC)
<i>Candida albicans</i>	*	*
<i>Citrobacter freundii</i>		
<i>Enterobacter cloacae</i>		
<i>Escherichia coli</i>	*	*
<i>Klebsiella pneumoniae</i>		
<i>Pseudomonas aeruginosa</i>		
<i>Serratia marcescens</i>		

Table 4. CFU determination of microorganisms without AGC and with AGC

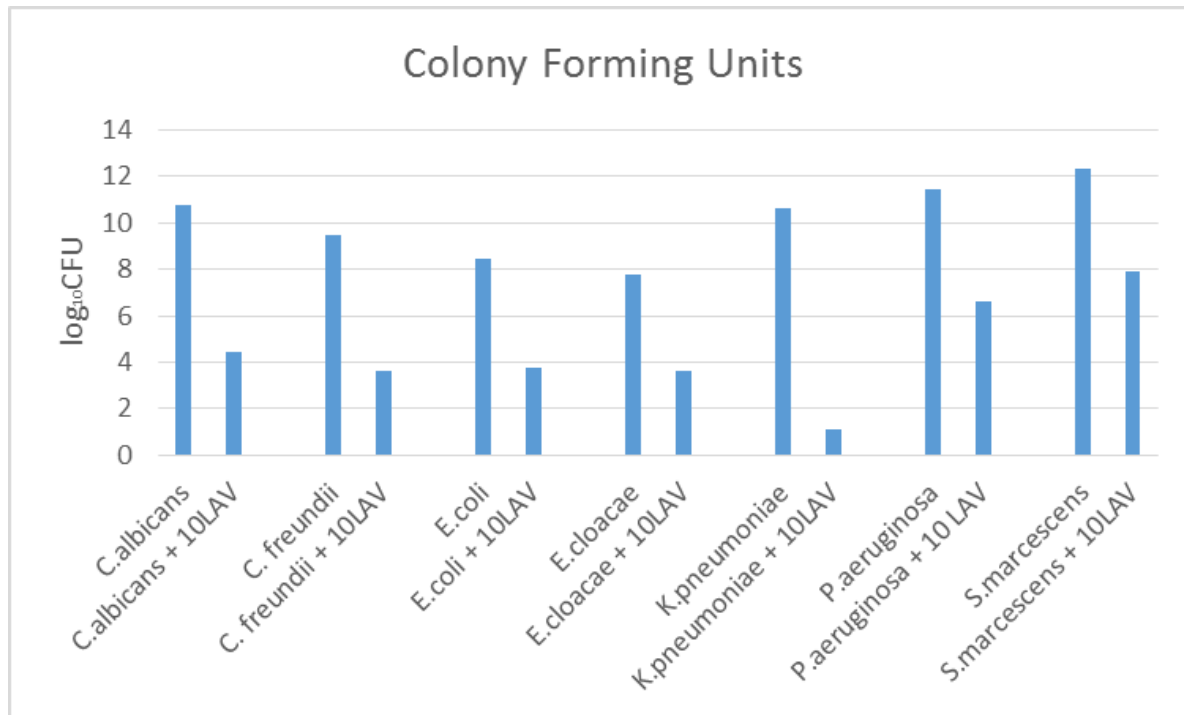


Figure 2. Logarithmic graph of CFU experiments of microorganisms grown with or without 10% AGC.

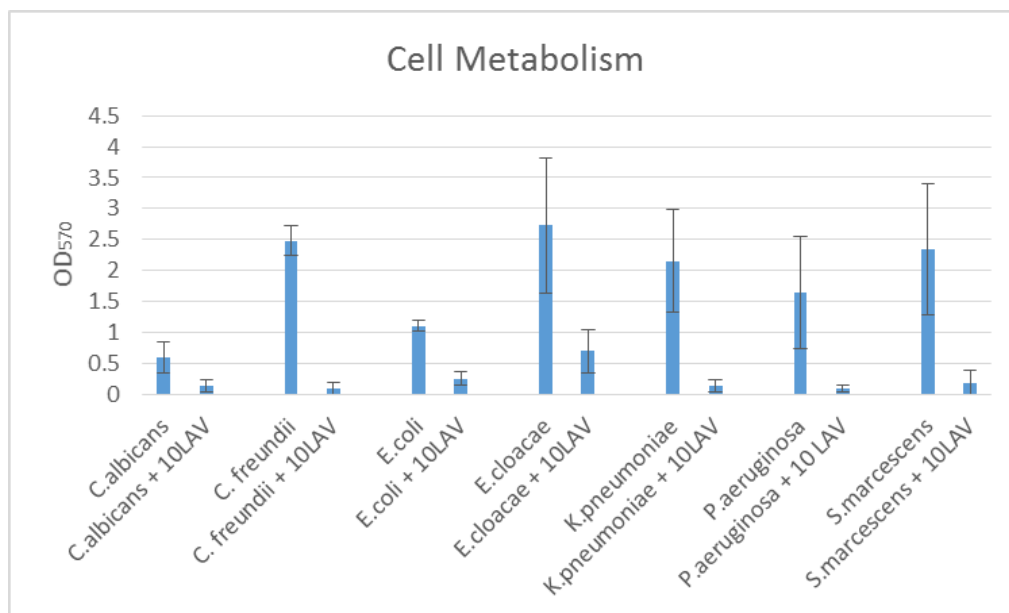


Figure 3. Graph of cell respiration using MTT assay on microorganisms grown with or without 10% AGC. Standard deviation bars shown.

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